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A NEW METHOD FOR LONG-TERM CULTURING OF INTESTINAL BIOPSIES DERIVED FROM CELIAC DISEASE PATIENTS USING MICE AS CARRIERS

Master's thesis

University of Tampere

BioMediTech (BMT)

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MASTER'S THESIS

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ABSTRACT

Celiac disease (CD) is a complex disorder with no bona fide animal model that would exhibit all the features of the disorder. The small-intestinal mucosal biopsies commonly used to study the disease present a limiting factor, which is the survival of the specimen for only 24-48 hours. The goal of this project was to develop a system to support the biopsy using mice as carriers for a long-term culture and to subsequently use this system to test potential therapeutic compounds. We obtained biopsies from CD and control patients and implanted them under the skin of athymic mice for the duration of 8 days. To determine if certain key features of CD were maintained in the biopsy during the extended incubation time, we performed various immunofluorescent stains. We found abundant, well developed vessels in the control and reduced size and functionality in the CD vessels, consistent with the situation in actual patients. We also confirmed the presence of B-cells, and found evidence of IgA/transglutaminase 2 colocalization, both important features of the disease. To test the effectiveness of different compounds at ameliorating the decreased vascularization seen in CD samples, we applied statin class drugs, shown to increase angiogenesis, to HUVEC cells in the presence and absence of celiac disease-specific transglutaminase 2 – targeting autoantibodies. We found that the compound α-amino-γ-butyrolactone hydrobromide had the ability to accelerate the growth of the HUVEC cells even in the inhibiting presence of the CD autoantibodies, while the drug Atorvastatin show variable and inconclusive results. We proceeded with the application of both of these compounds to mice implanted with CD and control biopsies. The samples from these final mouse experiments have yet to be analyzed but we feel confident that our organ culture model provides a valid option for extending the life of human biopsies as well as an opportunity to test novel compounds.

CONTENTS

ABBREVIATIONS

ABL α-amino-γ-butyrolactone-hydrobromide

APC Antigen presenting cell

cAMP Cyclic adenosine monophosphate

CD Celiac Disease

EBM Endothelial basal medium

ECM Extra-cellular matrix

EGM Endothelial growth medium

GDP Guanosine diphosphate

GFD Gluten free diet

GTP Guanosine triphosphate

HLA Human leukocyte antigen

HMG-CoA 3-hydroxy-3-methylglutaryl-coenzyme A

HUVEC Human umbilical vein endothelial cells

IEL Intraepithelial lymphocyte

IgA Immunoglobulin A

IgG Immunoglobulin G

IL-10 Interleukin 10

IL-15 Interleukin 15

INF-γ Interferon-gamma

MHC Major Histocompatibility complex

ROS Reactive oxygen species

TG2 Tissue transglutaminase

TNF-α Tumor necrosis factor-alpha

1 INTRODUCTION

Celiac disease (CD) is an autoimmune disorder affecting approximately 1 in 100 people world-wide and leading to the prescription of a challenging, gluten-free diet for life. This disease is characterized by the loss of the nutrient absorbing villi of the small intestine along with many classical symptoms in response to the consumption of dietary gluten. On a molecular level, it is customary to find increased levels of antibodies against the transglutaminase 2 enzyme (TG2) which is responsible for the post-translational modification of the toxic gliadin peptide and is also involved in cellular processes such as angiogenesis.

Angiogenesis is the generation of new blood vessels from pre-existing ones and is considered an important process in the maintenance of normal tissue as well as in the healing of damaged tissue, such as the small intestinal mucosal injuries suffered during the course of CD. The autoantibodies against the TG2 enzyme have been shown to disrupt angiogenesis at several steps including endothelial cell migration and sprouting (Myrsky et al. 2008), although the mechanisms for these effects remain unclear. Despite the elusiveness of these mechanisms, experimental evidence shows a significant role for the RhoB protein in these anti-angiogenic effects (Martucciello et al. 2012). This protein is functionally activated by the mevalonate pathway, which is most commonly known for the production of cholesterol, and due to its close association with the TG2 enzyme provides an interesting target for exploring the restoration of angiogenesis in tissues affected by CD.

To date, there is no model to encompass the full scope of cellular processes affected by CD and in turn, the testing of alternative treatment options is limited.

The purpose of this study was to develop a long-term organ culture system capable of representing the features of CD and offering the opportunity to test the effectiveness of different substances at ameliorating specific properties of the disease, such as the detrimental decrease in the generation and migration of endothelial vessel cells.

2 REVIEW OF THE LITERATURE

2.1 Overview of Celiac disease

Celiac disease (CD) is an autoimmune disorder that affects approximately 1-2% of the population worldwide, although experts estimate that due to widespread dietary and environmental change along with more focus on proper detection, that percentage will increase (Castillo et al. 2015). In Finland, for example, where screening for the disease is common and wheat and rye products are frequently consumed, the prevalence is about 2% (Lohi et al. 2007), although the prevalence is dependent on more than just the level of detection. The development of CD depends on a genetic predisposition of the human leukocyte antigen (HLA) Class II for the DQ2 or, in fewer cases, the DQ8 subtype, along with the ingestion of dietary gluten. In individuals predisposed with the HLA-DQ2 or 8 alleles, the presentation of CD is possible, but not without the trigger, gluten. There are many varied manifestations of the disease, the most common include malabsorption and chronic diarrhea which lend to anemia and weight loss. These are especially dangerous in children and can lead to malnutrition and stunted growth. The symptoms can differ between adults and children and typically vary from patient to patient as well. Although the most common symptoms are predominantly intestinal, there are extra-intestinal manifestations as well, including the skin disorder dermatitis herpetiformis, osteoporosis of the bones and gluten ataxia in the brain (Castillo et al. 2015). All of these symptoms are initialized by an inflammatory intestinal response to the ingestion of wheat, specifically to a component of the wheat gluten protein known as gliadin, or in general to the prolamine polypeptides of wheat, barley and rye (Castillo et al. 2015). The inflammation and destruction of the small-bowel mucosal architecture leads to a reduction in the area where nutrients can be absorbed and increased permeability of the surface of intestine, allowing the passage of more and more harmful gluten peptides and causing further destruction (see Figure 2.1).

In the intestine, the gliadin peptide undergoes post-translational modification by transglutaminase 2 (TG2) which results in a deamidated peptide with a higher affinity for the HLA-DQ2 or DQ8 molecule found on the antigen presenting cells (APCs) (Sollid and Jabri 2011).TG2 is found constitutively throughout the body and IgA class autoantibodies directed against this endogenous TG2 enzyme is what classifies CD as an autoimmune disorder. It is known that gliadin peptides are resistant to gastrointestinal enzymes because of their high glutamine content. Additionally, the amino acid sequences they contain bind specifically to HLA-DQ2, and the many glutamine residues provide ample substrate for TG2 to facilitate deamidation of these peptides to glutamic acid. This in turn provides Tcells with a very potent trigger, creating the perfect opportunity for this classic inflammatory response

(Shan et al. 2002). It has been established that CD4+ Tcell response, the central element of the adaptive immune response in CD, is strongly, albeit indirectly, affected by the inflammatory cytokine interleukin 15 (IL15) reacting to the toxic gliadin peptides (Maiuri et al. 2003). A study by Luciani et al. (2010) showed that the non-immunodominant gliadin peptide p31-43 was capable of inducing rapid expression of IL15 as well as enterocyte apoptosis, and subsequent addition of the immunodominant gliadin epitope induced Tcell activation (Luciani et al. 2010). All of this confirming the idea that while Celiac disease symptoms are dependent on adaptive immune response, it is with the innate immune response that the initial activation of the disease begins. Figure 2.1 offers a clear representation of the intestinal immune response in CD as it is currently understood.

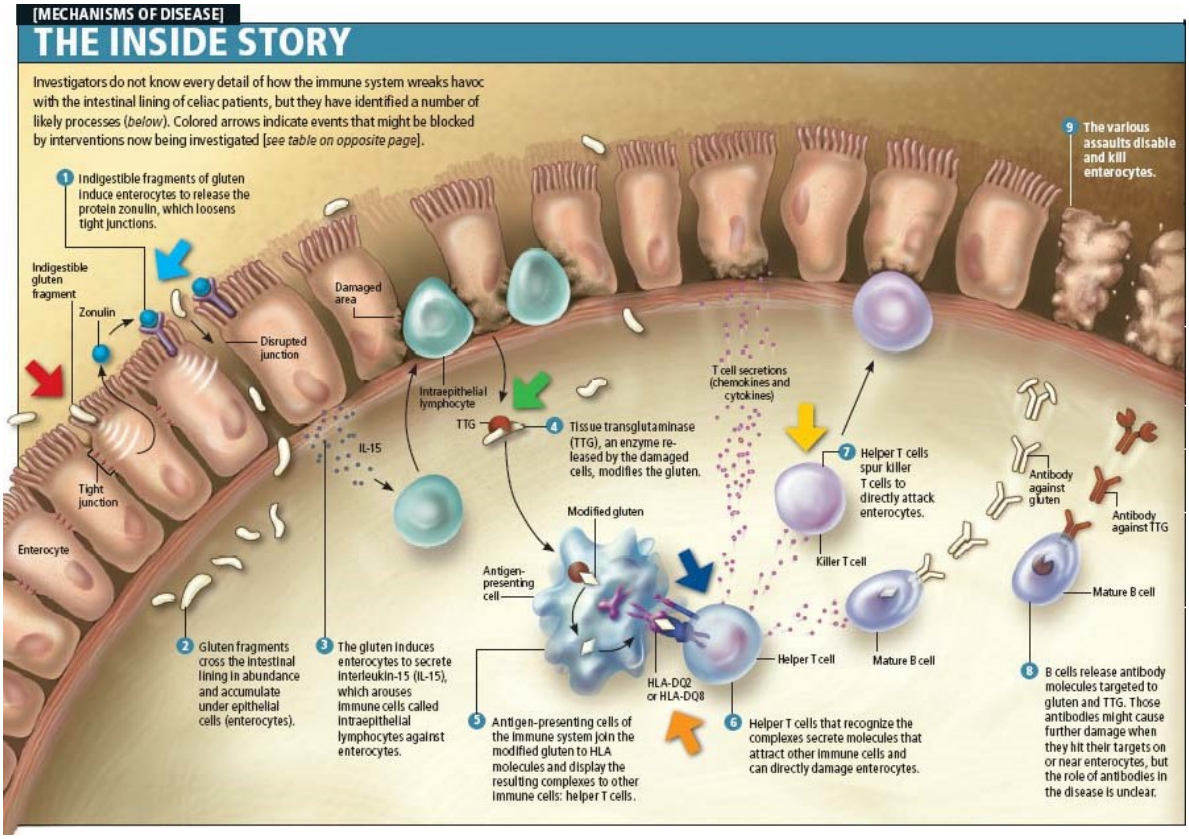


Figure 2.1: Dietary gluten is cleaved into peptides creating harmful gliadin peptides which cross the junctions of the intestinal barrier to the lamina propria. Once in contact with enterocytes, the gliadin stimulates the release of IL-15 which in turn induces intraepithelial leukocytes (IELs) to expand and begin their function of killing target cells, in this case enterocytes. TG2 modifies the gluten peptides for presentation as an antigen on the APC by the HLA-DQ2 or DQ8 molecule. The HLA-gliadin complex is recognized by helper Tcells which in turn recruit other immune cells such as natural killer Tcells which secrete additional cytokines and chemokines to further stimulate the destruction of the enterocytes, and Bcells which form and release antibodies against the gliadin peptides as well as TG2. These antibodies form deposits along the basement membrane and are also released into the circulating bloodstream. (Figure adapted from Sollid et al, 2009)

The diagnosis of CD can be extremely complex, due to broad spectrum of presenting symptoms, and distinct problems in diagnostics. The serological tests which are considered the initial step after suspicion of CD are extremely reliable and accurate, and normally these tests will detect IgA antibody levels against TG2 or de-amidated gliadin with greater than 90% accuracy (Castillo et al. 2015). Notwithstanding the essential role that serological tests play in the diagnosis, it is the intestinal biopsy showing flattened villi which has been considered the final word for confirming CD, although as recently as 2012, the guidelines for diagnosis in children were updated to exclude the necessity of a duodenal biopsy and rely solely on serology and histology along with optional DQ typing (Husby et al. 2012). Because of this, each intestinal biopsy is regarded as of great value and many physicians only take as many as are needed for clinical confirmation, often making the procuring of biopsies for study in the laboratory setting challenging. Because a gluten free diet can counteract all the symptoms associated with CD, and reverse positive serological results and injury to the mucosa, both the serological and intestinal biopsy methods of diagnosis require that the patient be on a diet containing gluten for at least one month to ensure detectable levels of immune response. For patients who are already on a gluten free diet and are hesitant to subject themselves to the discomfort of a gluten challenge for diagnosis, testing for genetic markers (HLA-DQ2 or DQ8) is a good option for exclusion, although, if they test positive for the markers, a biopsy would then be recommended. For these patients, alternative options for diagnosis also exist in the form of ex vivo gluten challenge, as described in the following studies. One study showed that the expression of cell surface receptor HLA-DR, when induced with gliadin, is a good diagnostic predictor of CD (Tortora et al. 2012). Another study measured the cytokine release in biopsies from both CD patients and healthy controls in response to gluten stimulation, and found that the differences in levels of certain cytokines between these groups, specifically TNF-α, IFN-γ, and IL-10, was sufficient for the confirmation of the disease (Vanga et al. 2014). Although it is still considered common practice to harvest several biopsies of the small bowel mucosa for confirmation of the disease, the follow-up biopsy after the patient has adhered to a gluten free diet for several months is no longer common, which makes the process of intestinal restoration during a gluten free diet (GFD) or during alternative treatments difficult to study. To date, a lifelong adherence to a GFD is the only approved treatment for a patient with confirmed CD.

2.2 Angiogenesis and the role of transglutaminase 2 in Celiac disease

In general, the process of angiogenesis is responsible for the formation of new capillary blood vessels from existing ones, through a tightly regulated balance of growth and inhibition, too much in either direction upsets the balance and leads to complications. In the case of CD, this process is essential to

the healing of the intestinal mucosa which is subjected to intensive damage throughout the course of the disease. The normal vasculature of the small bowel mucosa breaks down during the course of untreated CD which compromises the integrity of the endothelial barrier and thus contributes to the pathology of the disease (Myrsky et al. 2009). One of the results of CD is the formation of IgA class autoantibodies against endogenous TG2, which is one of the enzymes known to be involved in the regulation of angiogenesis (Caja et al. 2011). These antibodies are found as deposits surrounding the capillaries of the intestinal mucosa, and also flowing freely in the blood. In tissues, the antibodies are bound to the TG2 enzyme and the deposits of these antibodies co-localized with the enzyme are a classical hallmark of CD. In addition, the disappearance of these antibodies correlates with the healing of the intestinal mucosa during the patient's adherence to a GFD (Koskinen et al. 2010).

The formation of new blood vessels relies on the behavior of the endothelial cells that form them; the proliferation, migration and differentiation of these cells are essential to the angiogenesis process. TG2, of which endothelial cells have an abundance, assists in the stability of the ECM by crosslinking the proteins located there and by providing this stability could facilitate the formation of new vessels, although the mechanisms are still under investigation (Jones et al. 2006). In fact, this same study discusses the possibility that TG2 can both promote and inhibit angiogenesis depending on the abundance, suggesting a regulatory role for the enzyme. TG2 also has additional roles including those in wound healing, cell adhesion and cell death. The study by Myrsky et al., in 2008 suggests that IgA class antibodies targeted against TG2 are not merely a result of the disease but actually a cause of its pathogenicity. This is supported by evidence that the antibodies produced in the small bowel have the ability to bind recombinant TG2, induce intestinal epithelial proliferation but inhibit differentiation of the same, and also weaken the epithelium making it more permeable (Myrsky et al. 2008). These results suggest that the autoantibodies are indeed themselves pathogenic, which is in contrast to the widely accepted theory that they are simply a product of the disease. The vascular arrangement within the small-intestine is important not only structurally, but also in terms of digestion and absorption of nutrients. Normally, TG2 is involved in the regulation of the organization of the cytoskeleton, and so during the course of the disease, autoantibodies against the enzyme cause a disruption in the actin cytoskeleton (Myrsky et al. 2008). The antibodies are found bound to TG2 on the capillary cells, both endothelial and vascular mesenchymal, and prevent the sprouting and migration of these cells, and these defects are thought to be due to the disorganization of the cytoskeleton (Myrsky et al. 2008). Within a year of showing that angiogenesis is inhibited by CD antibodies, Myrsky et al., continued to demonstrate in their 2009 study that these antibodies also increase the permeability of the blood vessels

of the small bowel mucosa. The study provided evidence that both IgA and IgG antibodies derived from CD patients increased not only the permeability of the endothelial monolayer, but also the adhesion of lymphocytes to these endothelial cells, possibly through the action of the E-selectin adhesion molecule as this was found to be upregulated in the same experiments. It is suggested by the authors that the increased adhesion of lymphocytes could be in response to the IgA class autoantibodies that are also found bound to the endothelial cells, with the end result being an increase of lymphocytes migrating across the endothelial barrier. Additionally the study suggested that CD patient IgA increases the activity of TG2 in endothelial cells, at least the transamidation activity contributing to crosslinking of proteins in the extra-cellular membrane (Myrsky et al. 2009).

2.3 Small GTPases regulate vascular biology

The Myrsky et al. study from 2009 also investigated the relationship between the small GTPase RhoA, and TG2. RhoA is known to regulate the actin cytoskeleton and is also involved with the permeability of the endothelial barrier (Spiering and Hodgson 2011). TG2 has been shown to be an activator of the small protein and this interaction was therefore of interest in the context of CD (Janiak et al. 2006). The question was, since TG2 regulates the organization of the cytoskeleton and autoantibodies against the enzyme can cause disruption of the cytoskeleton and increase the permeability of the endothelial blood vessels, can these same antibodies activate the RhoA protein? The result was that the IgA antibodies derived from CD patients undoubtedly have the ability to activate the RhoA protein which then increases the endothelial permeability, and that the inactivation of this protein prevented the dysfunction of the endothelial barrier in terms of permeability and lymphocyte migration. These results also established that the TG2 enzyme and the RhoA protein were both operating within the same pathway, which is activated by the CD IgA antibodies.

A review in 2010 by Spindler et al. summarizes the roles of different GTPases including RhoA, in the regulation of vascular permeability, and relates their activity to angiogenesis and inflammatory states. Although Rac1, Cdc42, and RhoA from the Rho GTPase family, and Rap1 from the Ras GTPases are the focus in this review, there are other players coming to light as regulators of the endothelial barrier integrity. GTPases are only active to advance signaling pathways when they are bound to GTP; after the phosphate group is cleaved to yield GDP, they are considered to be in their inactive state. Because all of these proteins mentioned above are considered small GTPases, they do not possess strong abilities to hydrolyze GTP and therefore, their activation and deactivation are controlled by a group of proteins which either enhance or restrict their activity (Spindler et al. 2010).

During an inflammatory response, an increase in vascular permeability is seen as the tight junctions between endothelial cells relax to allow the passage of the extra fluid that accumulates. Rho GTPases in general are known to play a role in the regulation of these changes in cellular adhesion through reorganization of the actin cytoskeleton, and whereas Rac1 and Cdc42 both maintain endothelial barrier integrity, RhoA was found to destabilize the tight junctions through contraction of the stress fibers (See Figure 2.2)(Spindler et al. 2010). Rac1 was determined as essential for the integrity of the endothelial barrier under resting conditions, through several experiments which limited the activity of the protein with different bacterial toxins (Spindler et al. 2010). Additional tests were performed on Rac1 and other RhoGTPases such as RhoA and Cdc42 to determine if they would have the same stabilizing effect under inflammatory conditions, and it was found Rac1 activation decreases vascular permeability in part through the barrier protective properties of the cAMP signaling pathway (Spindler et al., 2010). The other enzyme equally relevant to barrier maintenance is Cdc42 and the Spindler et al., 2010 review highlights that the cAMP pathway activates both Rac1 and Cdc42 similarly and as a result their functions are hard to differentiate. However, deeper investigation has revealed that Cdc42 is important not only for barrier maintenance, but also for its restoration. The authors suggest that the disruption of adherens junctions is a possible trigger for the activation of Cdc42 but that its activation could be dependent on the type of disruption that the barrier suffers (Spindler et al. 2010).

RhoA, in contrast to both Rac1 and Cdc42, is clearly involved in the reduction in endothelial barrier stability, and the inhibition of the Rho kinase reduced permeability of the endothelial barrier. Rho kinase is a known mediator of the myosin light chain and the end result is inactivation of the MLC phosphatase which then leads to an increase in the phosphorylation of the MLC and in turn increases the contractility of actin-myosin by the formation of stress fibers (Spindler et al. 2010). It is suggested that actin-myosin contractility actually pulls apart the membranes of neighboring cells, contributing to barrier destabilization; although admittedly, this result was only shown in *in vitro* studies and the same result was not obtained *in vivo*. Overall, it appears that the regulation of cell junctions by Rho proteins and their kinases may occur through disruption of either the actin cytoskeleton or the tight junctions.

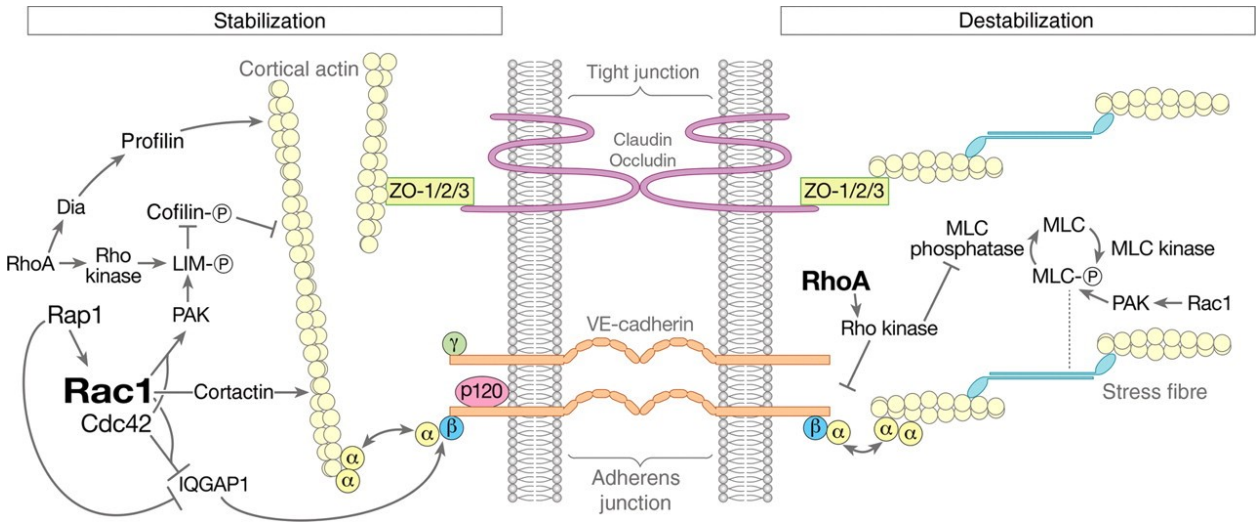


Figure 2.2 GTPases regulate barrier function in different ways. Rac1 and Cdc42 are thought to stabilize the barrier (left) while RhoA is thought to destabilize (right) through the contraction of stress fibers and through the interruption of VE-cadherin adhesion. (Figure source Spindler et al, 2010)

2.4 RhoB and its role in angiogenesis

Another protein that is of interest is also a small GTPase from the Rho family known as RhoB. Its functions include roles in receptor mediated endocytosis as well as the expression of certain vascular genes, and additionally a role in apoptosis (Stamatakis et al. 2002).

RhoB can be post-translationally modified at the C-terminus by either farnesylation or by geranylgeranylation and it is suggested that the isoprenoid group that is added by either of these modifications is key to the function of this protein (Stamatakis et al. 2002). The regulation of the levels of RhoB is under the control of the mevalonate pathway, better known as the cholesterol pathway for its synthesis product. In the 2002 study mentioned above, the authors demonstrated that downregulation of this pathway leads to an increase in the amount of both the mRNA and protein levels of RhoB, more so in the protein levels. They propose that the isoprenylation of the protein, for which the mevalonate pathway provides the intermediates, is essential for the degradation of the RhoB protein and therefore, for the regulation of its cellular levels. The modification of this protein can be prevented by any farnesyl transferase inhibitors or by a drug that blocks the mevanolate pathway, the most popular being the cholesterol lowering drug known as simvastatin (Stamatakis et al. 2002). A more detailed look at the effects of this class of drugs will be given in the next section.

In the context of CD, RhoB expression is upregulated at both mRNA and protein levels in response to CD patient total IgA and to CD patient derived antibodies specific to TG2, which are proven inhibitors of angiogenesis (Myrsky et al. 2008). Down regulation of RhoB by siRNA was able to reverse the

effects of the anti-TG2 antibodies (Martucciello et al. 2012). Although it is known that the active form of TG2 activates RhoA, and that RhoA can regulate the activity and expression of RhoB, the authors postulate that a mechanism other than RhoA mediated regulation is responsible for the upregulation of RhoB seen in response to CD patient IgA.

Previous studies like the one in 2003 by Adini et al., have explored the role of RhoB in vascular development. The process of blood vessel formation from endothelial cells is known as vasculogenesis and has different properties than the expansion of existing vessels, what is known as angiogenesis. Angiogenesis can occur through different routes, both expansion and sprouting, and is of interest in the context of CD, especially in this section regarding RhoB. Sprouting angiogenesis, which is thought to be the major source of new blood vessels during development and wound healing, is the formation of new vessels from existing ones and it requires both cell migration and tubule assembly. This study by Adini et al., in 2003 provides a link between RhoB and the Akt pathway, specifically the branch of the pathway involved in cell survival and apoptosis. The Akt survival pathway generally inhibits the expression of cell death genes while enhancing the expression of those genes for anti-apoptosis. The Akt was shown to accumulate in and around the nucleus of sprouting endothelial cells, colocalized with the RhoB protein, and depletion of RhoB led to the exclusion of Akt from the nucleus indicating the importance of this colocalization. The study also showed that depletion of RhoB leads to apoptosis and tubule formation defects in endothelial cells during the formation of new vessels, leading the authors to conclude that RhoB plays a role in the regulation of endothelial cell survival during vascular development (Adini et al. 2003). As previously mentioned, the Martucciello et al. study from 2012 shows that the up-regulation of RhoB in response to CD patient autoantibodies against TG2 and the corresponding loss of angiogenesis could be ameliorated by the inhibition of the synthesis of RhoB. The same study suggests the role of the farnesylated RhoB in modifying the actions of CD autoantibodies, and considers the interruption of the mevalonate pathway with drugs as a possible therapeutic control.

2.5 Statin class of drugs and their effect on angiogenesis and other CD related functions

Statin drugs have in history been used mainly for their cholesterol lowering properties, but in recent years it has become apparent that these drugs can be used in many other ways, including to control vascular inflammation and endothelial function (Gazzerro et al. 2012). Statin drugs function through the mevalonate pathway by binding and inhibiting HMG-CoA reductase and in this way act as an inhibitor throughout the rest of the pathway, including for such enzymes as Farnesyl transferase and

Geranylgeranyl transferase (See Figure 2.3), whose importance for activation of Rho GTPases was highlighted in the previous section. Although many variations of statins exist, the few that have gained the most attention are Simvastatin, which is the most well-known, Atorvastatin and Lovastatin. The difference between these compounds is in their slight structural variations along with the organisms they were isolated from, although their mechanism of action is similar to each other. Their structure lends to the affinity with which they bind their target, the HMG-CoA reductase, and they are classified in this way.

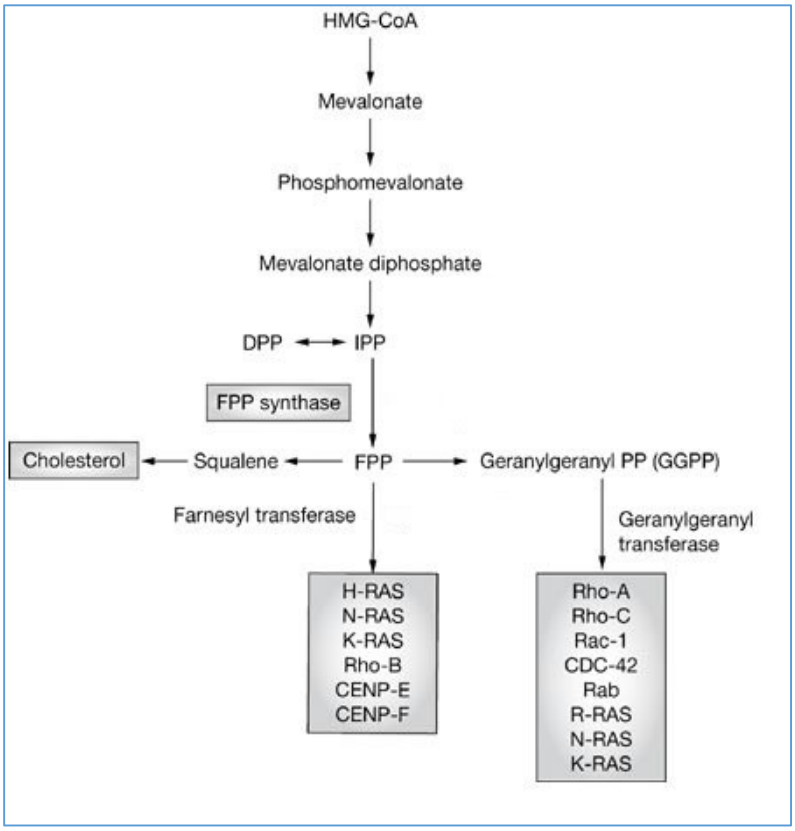


Figure 2.3 The Mevalonate pathway is important for the biosynthesis of cholesterol and can be interrupted early by the binding of statin class drugs to HMG-CoA reductase, or later by farnesyl transferase inhibitors or geranyl-geranyl transferase inhibitors. The inhibition of these steps can prevent the modification of RhoB, which is essential for its degradation. Figure modified from: (Santini et al. 2006)

According to the Gazzerro 2012 review, several studies have shown that these drugs modulate the immune system in addition to their effect on cholesterol synthesis. The main modulation occurs through the interruption of the communication between the MHC molecule (both class I and II) and CD4 and CD8 Tcells, preventing their activation. Statins also have an effect on the costimulatory

action of endothelial, dendritic and lymphocyte cells including those induced by IFNγ. Although an immunosuppressive response is implied by this knowledge, further study is needed to clarify the extent to which this reaction is elicited by statin drugs. Another interesting statin induced cellular response is the inhibition of adhesion molecule LFA-1 which is important for Tcell-Tcell interactions, and leukocyte infiltration and migration. It is interesting to consider these responses to the administration of statin drugs in the context of the inflammation of the mucosa in the course of CD. Additionally, the Gazzerro 2012 article highlights the well-established statin induced effects seen on endothelial cells, which include the increase of angiogenesis and therefore the improvement of vascular protection and the function of the endothelium, especially in terms of brain and heart tissue. In contrast, the same 2012 review discusses the evidence for the reduction of angiogenesis in tumors with the application of statins, revealing the dual nature of these drugs, which is highly dependent on the dose. Low dose statin application (<0.05μM) *in vitro* was shown to increase angiogenesis while doses higher than that have the opposite effect, inducing apoptosis and the down-regulation of VEGF; the same pattern was also found in animal models.

Arguably one of the most interesting effect of statins in terms of diseases other than the cardiovascular ones, is the ability of all statin drugs to reverse endothelial dysfunction through various actions including Akt activation and prevention of Rho prenylation (Gazzerro et al. 2012). Some evidence discussed in this same review also showed inhibition of apoptosis of endothelial cells and the subsequent contribution to the healing of the endothelium after inflammation and injury. Several studies support the ideas in the 2012 review by Gazzerro et al and they are presented below.

One of these, a study by Soehnlein et al. in 2004 shows evidence of statin drugs positively affecting the stability of the basement membrane, which, in moderation can be advantageous to endothelial function in the context of new blood vessel formation. They showed that when Atorvastatin was administered to HUVEC cells, a dose dependent increase of TG2 expression was seen, and that this increase was not due to an increase in endothelial cell apoptosis, which is also known to up-regulate this enzyme. It is known that TG2 plays an important role in the stabilization of the basement membrane and therefore an increase in the enzyme after endothelial dysfunction could be considered beneficial in some cases. Conversely, a study by Jones et al. in 2006 showed that the application of TG2 to *in vitro* and *in vivo* angiogenesis assays inhibited endothelial tube formation and furthermore, suggest that this inhibition could be useful in the context of suppressing tumor growth.

An additional study investigated the use of Atorvastatin in rats with restricted blood flow to their hind limbs and found that low doses of the drug improved blood flow to the area, and increased pro-angiogenic cytokines such as Ang-1and 2, VEGF and IL8 (Matsumura et al. 2009). Another related study in 2010 found that the application of Simvastatin after traumatic brain injury in rats increased endothelial cell proliferation and tubule formation. The authors suggest that this effect of Simvastatin is through the activation of the Akt signaling pathway that was discussed earlier in this document (Wu et al. 2011).

A study in 2013 with Atorvastatin showed that this drug was capable of preserving the integrity of the endothelial junction through the modulation of small GTPase function (Xiao et al. 2013). As discussed previously, small GTPases have been shown to activate myosin light chain kinase, which plays a role in the constriction and relaxation of the cell, important to the barrier function and junction integrity. The Xiao et al. study shows that GTP loading, the process of converting the inactive GDP bound GTPase to the active GTP bound GTPase, and lipidation, which involves either the geranylgeranylation or farnesylation of the enzymes, are essential for their cell regulation. GTP loading was found to be increased in the presence of the drug while lipidation, which is essential for the cell membrane association and in turn the stress fiber formation by the GTPases, was inhibited. Both of these responses were associated with a decrease in inflammation and an increase in endothelial barrier integrity, although the mechanisms still remain elusive. Another important discovery that came from this study was that Rho and Rac were the two small GTPases involved in the inhibition of inflammation and the preserved endothelial junctions that the group observed in response to Atorvastatin (Xiao et al. 2013).

In the context of CD, it is also interesting to consider a study from 2005 about the response of intestinal T cells specific for gluten along with intestinal biopsies from CD patients, to Atorvastatin. This study by Ráki et al., first investigated the response of T cells derived from patients and specific for deamidated gluten, and found a decreased proliferation of the cells in response to gluten in the presence of Atorvastatin; a reduction in proinflammatory cytokines was also seen. The response of dendritic cells was also measured since they are the primary APCs for proper activation of T cells in CD. A dose dependent increase in apoptosis was found in dendritic cells when they were exposed to Atorvastatin, as in the studies discussed earlier, higher concentrations caused more apoptosis whereas the lower concentrations had little effect (Raki et al. 2005). Normally, dendritic cells display an increased expression of costimulatory molecules after activation, however, in the presence of Atorvastatin this expression was reduced. Additionally, this study included the application of Atorvastatin to intestinal

biopsies from CD patients, and measurement of the response by secreted cytokines. Unlike the T cells, biopsy secreted cytokines did not change significantly in response to Atorvastatin.

As is apparent from the many studies regarding the modulatory effects of statin class drugs on many cellular processes, it is worthy to consider them beyond the scope of cardiovascular disease as having potential for alternate disease targets. For CD, a disease in need of alternative therapies, statin drugs are valid options for further study considering their roles in modulating major factors in the disease such as the function of small GTPases and T cell activation.

2.6 Model systems for Celiac disease

2.6.1 Cell based models

Regarding the study of CD in cellular systems, both cell lines and intestinal biopsy organ culture exist, but to date there are no animal models that fully capture all the features of the disease. Due to the heavy burden of maintaining a gluten free diet, which is currently the only approved treatment for CD, an alternative treatment would greatly benefit those whose lives are effected by the disease. Clearly, *in vitro* and *in vivo* models for the disease are essential to study the molecular mechanisms at play, prior to the introduction of pharmaceutical interventions. Several of these models are presented below.

Of primary importance to the understanding of CD function is further investigation into the compromise of the epithelial barrier seen during the course of the disease. Several cells lines exist for this type of study including T84 and Caco-2 cells, both of which are derived from human colon carcinoma cells and used to study similar but slightly varied features of epithelial barrier disruption. T84 cells represent a well-established line for studying innate response to toxic gliadin peptides such as ROS and apoptosis (Maiuri et al. 2005, Luciani et al. 2010). These cells show increased proliferation, decreased differentiation (Halttunen and Maki 1999) and overall increased permeability in response to CD antibodies (Zanoni et al. 2006). T84 cells have also been used to highlight the role of IFNγ in the passage of gluten peptides through the epithelial barrier. Experiments performed by applying IFNγ produced by CD specific T cells to epithelial cell monolayer resulted in an increase of the movement of gluten peptides across the epithelial cell monlayer (Bethune et al. 2009).

More widely used than the T84 line for the study of CD is the Caco-2 cell line. Similar to the T84 line, these cells show enhanced proliferation and inhibited differentiation in response to gliadin, but additionally present with actin reorganization and apoptosis. This cell line has also been used to show how different CD factors (e.g. IFNγ, TNFα and gliadin) affect epithelial junctions, as well as to demonstrate the process through which gliadin is processed in the intestinal epithelium. Caco-2 cells

have also been used in testing various treatments for CD such as a tight junction modulator, gliadin binding agents and probiotics among others (Lindfors et al. 2012).

HLA DQ2 specific T cell lines are another important model for CD and were discussed in the previous section in reference to treatment with Atorvastatin. These cell lines have been especially useful in identifying the epitopes of gluten, secalin and hordein (from wheat, rye and barley respectively) that cause a T cell response (Lindfors et al. 2012).

The disadvantage of using cell lines is that they contain only one type of cell and therefore miss the signal interplay that exists in tissues and organs in the human body. Each of these attempts have succeeded in clarifying different aspects of the disease and are useful in their own way but naturally, progression towards a more complete model is needed.

One very interesting technique that could be useful in addressing some of the issues facing work with single cell lines is organoid culture. These offer the opportunity to study a three dimensional model, and the intestinal organoid would be especially useful in the study of CD. Sato et al. describe a method whereby intestinal crypts can be grown using only $Lgr5^+$ stem cells which undergo crypt fission and offer villus-like domains, both of which are essential features of the intestine that are affected during the course of CD (Sato et al. 2009).

2.6.2 Organ culture and animal models

It is therefore important to include here a discussion about organ culture and animal models of CD. Ideally, true patient material in the form of intestinal biopsies could be used to study features of the disease such as villous atrophy, crypt hyperplasia, and membrane disruption along with the secretion of cytokines and other factors. Although these features are measurable after extraction of the biopsy, there is a time limit on the viability of the tissue due to lack of blood supply, usually 24-48 hours. The organ culture of intestinal biopsies has nonetheless been extremely useful, especially in the description of the role of IL-15 in CD (Lindfors et al. 2012). The majority of studies with the biopsies in organ culture revolve around pathogenesis of the disease and the field would benefit greatly from an animal model to study more closely the progression of the disease in the presence of gluten, and also the healing of the intestinal mucosa during the course of a GFD or with the application of novel therapies.

Several attempts have been made at creating a complete animal model featuring all the necessary components of the disease, each with its own advantages and drawbacks.

A review of animal models of CD highlights the importance of understanding the complex interactions of the innate, adaptive and autoimmune response that leads to the development of CD, which has been more available to us through animal models (Marietta et al. 2011). Among the models that exist today, mice are the most varied with both "spontaneous" and genetically engineered strains. The "spontaneous" mouse models include those that develop symptoms of the disease, such as villous atrophy and crypt hyperplasia, in response to a gluten containing diet, but the reactions lack dependence on TG2 antibodies (Maurano et al. 2005) and (Sblattero et al. 2005). Other efforts towards a sufficient mouse model include transgenic mice that expressed HLA-DQ8 or DQ2 which each provided insight into the development of CD despite not presenting with classical symptoms of the disease (Black et al. 2002) and (de Kauwe et al. 2009).

Some models of larger mammals and even a few non-human primates have been created as summarized in Marietta et al., 2011. Irish setters were the first animal model for CD, and in these dogs they could induce flattened villi and IEL infiltration with a wheat diet but these symptoms were not dependent on MHC II of the animal. It is however an appropriate model to study some features of the innate immune response.

Another important animal model is the rhesus macaque, which spontaneously develops villous atrophy and antibodies against TG2 and gliadin on a wheat diet. The incidence of increased antibodies in the monkeys is about the same as it is for CD in humans, but the symptoms did not revert when a GFD was applied. The importance of this model lies with the ability to test novel treatments and also for the supply of healthy control biopsies that they provide, which are notoriously difficult to obtain (Marietta et al. 2011).

In conclusion, although each of these models provide access to information about different parts of CD pathology, none of them encompass they whole picture. Therefore, the search continues for an animal model appropriate for studying not only disease progression, but also alternate treatment options and the recovery process.

Consequently, organ culture is widely used to study different features of CD, with different advantages and limitations than either the cell models or the animal models. Organ culture has been used to simulate GFD conditions, and pathogenesis upon the introduction of gluten or gliadin to the culture. It is also possible to test different therapeutic compounds with this system and in this way bypass the need for expensive, and sometimes difficult to obtain, laboratory animals. In general, the small bowel mucosal biopsy culture has not changed significantly since it was introduced by Browning et al., in

1969. They introduced a system whereby the biopsy was suspended on a wire mesh bed supported by cotton to allow the exchange of culture medium and secretions to and from the biopsy (Browning and Trier 1969). Since then, this system has been used to investigate different aspects of CD, including the characteristics of the different cell types associated with the biopsy, and the role of IL-15 and other cytokines during the progression of the disease (Maiuri et al. 2000). One important limitation to this system is the time which the biopsy can survive outside of the body (24-48hr), which only provides a very brief window into the multi-faceted nature of the disease. Additionally, organ culture is limited in its representation of a living system since it lacks the complexity found in a living body. The scientific community and specifically the field of CD research would benefit from a more complete system that mimics a living body more closely and possesses certain essential features such as a circulatory system, with the purpose of extending the survival time of the biopsy for more complete studies.

3 AIMS OF THE RESEARCH

The aims of this study were 1.) To develop an organ culture system using mice as hosts with the purpose of extending the survival of a human small bowel mucosal biopsy for more than 48 hours while preserving key features of CD, and 2.) To use this system to test several compounds for their effectiveness at ameliorating different pathologies of CD, especially the decrease of angiogenesis seen during the course of the disease.

4 MATERIALS AND METHODS

4.1 Small bowel mucosal Biopsy culture in mouse

4.1.1 Culturing biopsies in matrigel plug

Prior to the start of this thesis project, intestinal mucosal biopsies were collected from 4 children. All samples were used with written parental consent and experimental procedures were approved by the Ethics Committee of the Tampere University Hospital, Tampere, Finland. Of the 4 samples, 3 were confirmed as positive for CD, and 1 was excluded for CD and used for an experimental control. 2 of the CD confirmed biopsy samples were too small for individual use and were combined for a usable amount.

These samples were segmented with a scalpel and mixed with BD matrigel matrix (BD biosciences, Bedford, MA, USA) and 1µg/µl of erythropoietin (EPO, Sigma Aldrich, St. Louis, Missouri, USA), and were then injected subcutaneously into the backs of 4 week old NUDE mice (Harlan Laboratories Inc. Horst, the Netherlands). Following an 8 day incubation period, the matrigel plugs were removed from the mice, suspended in Tissue-tek cryostat sectioning matrix (Tissue-tek O.C.T., Sakura, Flemingweg, the Netherlands) and snap frozen, prior to storage at -80°C for future analysis. The mice were treated according to Finnish and European Union regulations (62/2006 and 36/EEo/2006 for Finland, and 86/609/EC) and procedures were approved by the Finnish Authorities (ESAVI -2010-06223/Ym-23 and ESAVI/4279/04.10.07/2013) and by the Turku Central Animal Laboratory, Turku, Finland.

4.1.2 Processing of matrigel samples

Each frozen OCT block derived from the matrigel plug assays containing the matrigel/biopsy mixture was sliced onto glass slides. The slides were arranged in sets of 7 sequential slides containing 12 slices on each slide (see Figure 4.1) for coverage of the entire block. Slicing was carried out at -16°C to maintain the integrity of the OCT and tissue, each slice was 10 microns in thickness and all the blocks were sliced until no trace of the plug remained. Slides were store at 20°C until staining could be carried out.

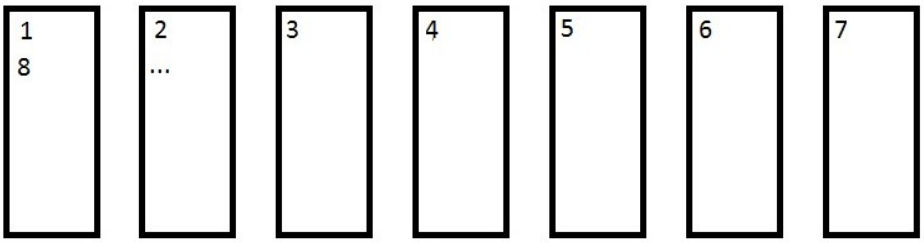


Figure 4.1 The OCT blocks were sectioned onto glass slides in a sequential manner with 12 samples on each slide. Each slide represents a different set which was subjected to different stains as described in the sections below.

4.1.3 Hematoxylin/Eosin stain and selection

Set 1 of the slides was stained with a standard Hematoxylin/Eosin protocol, to determine the presence of the matrigel/biopsy in the cryosliced samples. Hematoxylin stains the nucleus of cells blue, while eosin stains the cytoskeleton and extracellular proteins with an orange hue. Since matrigel is composed largely of extracellular proteins such as collagen, the HE staining method is appropriate for the detection of this material. The replicates of the slides in which portions of the biopsy or matrigel was present, were selected for different types of immunofluorescent staining, with a total of 31 slides for each stain type (16 CD+ and 15 controls).

4.1.4 Stain for von Willebrand Factor and quantification of vessels

One entire set of slides from the matrigel plug assays was stained with anti-Von Willebrand Factor (vWF) to determine the presence of blood vessels in and around the pieces of biopsy embedded in the matrigel. vWF is a large glycoprotein known to bind other proteins such as collagen, but does not have any catalytic effect. It is constitutively produced in connective tissue and the endothelium (Sadler 1998). For the immunofluorescent staining of the slides, they were brought to room temperature for 5 min. then fixed in 10% neutral formalin (Sigma-Aldrich, St Louis, MO, USA) for 15 min. at 4°C. After fixation, the slides were washed 3 times for 5 min. in PBS followed by 30 min. in a blocking solution of 0.2mg/ml BSA (Sigma-Aldrich) and goat serum (DAKO) (1:20) in PBS. Following blocking, the slides were incubated with rabbit-anti vWF* (1:250 Dako, Glostrup, Denmark) for 30 min. at 37°C and counterstained with goat-anti-rabbit Alexa 488 (1:1000 Molecular Probes, Eugene, OR, USA), also for 30 min. at 37°C. The slides were mounted with Vectashield mounting media containing DAPI stain (Vector laboratories, Burlingame, CA, USA). Images were taken with an Olympus BX60 microscope

(Olympus Europa GmbH, Hamburg, Germany) and CellD image software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany). The size and number of blood vessels in and around the biopsies and matrigel were quantified using the ImageJ software (Rasband W.S., ImageJ, US. NIH, Bethesda, Maryland, USA)

*The primary anti-vWF antibody is known to cross react with mouse, any vessels present could possibly be derived from the human biopsy or the host mouse.

4.1.5 Stain for CD20

Another set of slides from the matrigel plug assays was stained for the presence of the CD20 antigen on human B-lymphocytes, in the same way as described above, first using a goat-polyclonal antibody against CD20, specific to human and not cross reactive with mouse, (1:50 Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by chicken-anti-goat with Alexa 594 (1:1000 Molecular Probes). The slides were again mounted using the Vectashield with DAPI stain and image capture and processing were carried out in a similar fashion.

It is important to note that an attempt was made to detect the CD3 antigen on human T lymphocytes in a double stain with the CD20 antibody, but all experiments with the polyclonal rabbit antibody against human CD3 (1:50 Santa Cruz) failed to produce any immunofluorescent signal.

4.1.6 Stain for TG2 and IgA colocalization

To determine if the classic CD-characteristic colocalization of CD patient IgA antibodies against TG2 and the TG2 enzyme itself were preserved in the biopsy implanted in the matrigel plug, a double stain was done on 1 set of the slides described in section 4.1. The slides were subjected to the same fixation and blocking method described in section 4.1.4 using primary antibodies FITC-conjugated anti-human IgA (1:40 DAKO), and polyclonal rabbit-anti-human TG2 (1:20 Zedira GmbH, Darmstadt, Germany). The TG2 antibody was counterstained with goat-anti-rabbit with Alexa 569 (1:2000 Molecular Probes) and the slides were again mounted with Vectashield medium and imaged in the same way as previously described.

4.2 HUVEC culture

Human umbilical vein endothelial cells (HUVECs Clonetics, San Diego, CA, USA) were cultured in endothelial basal medium-2 (EBM-2 Clonetics) supplemented with the endothelial growth medium-2 bulletkit (EGM-2 bulletkit Clonetics) at 37°C and 5% CO_2.

4.2.1 Cell-IQ with 5 statins

To test the response of HUVEC cells to the presence of statin class drugs, the cells were seeded into a 24-well plate and grown to confluency in EGM-2. Two different concentrations of the following 5 drugs; α-amino-γ-butyrolactone hydrobromide, pravastatin, lovastatin, mevastatin, and atorvastatin, were added in separate wells, following which, the cells were subjected to a scratch wound using a sterile 20µl pipette tip (see Figure 2). The plate was incubated in the Cell-IQ system (Chipman Technologies, Tampere, Finland) where cell migration into the wound area was tracked continuously for 64 hours, and the percent closure of the wound in each well was calculated.

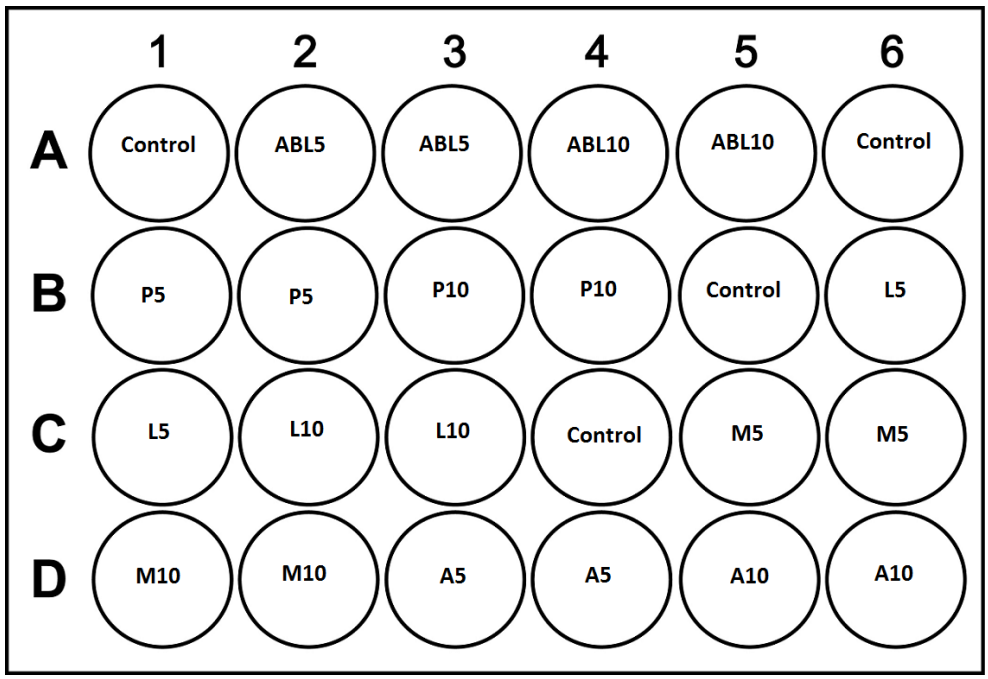


Figure 4.2 Control wells contained only EGM2, drugs were added to wells for the final concentrations as follows: α-Amino-γ-butyrolactone hydrobromide (ABL) 5 and 10µM, Pravastatin (P) 5 and 10µM, Lovastatin (L) 5 and 10µM, Mevastatin (M) 5 and 10µM, Atorvastatin (A) 5 and 10µM. The scratch wound was performed horizontally in the right to left direction.

4.2.2 Cell-IQ with 2 statins

A second group of HUVEC cells were treated as previously described, with the additional use of an antibody derived from CD patients against TG2 major celiac epitope, referred to in this experiment as 4.1 (described in Simon-Vecsei Z. et al., 2012 and Di Niro R. et al., 2007). Concentrations of 50 and 100µM ABL, 10 and 20µM Atorvastatin, and the absence and presence of the 4.1 minibody were used in this assay. Cell migration into the scratch wound was again measured over the course of 64 hours with the Cell-IQ system. The statins and 4.1 minibody were added in the following order; ABL100, ABL50, ABL100+4.1, ABL50+4.1, At20, At10, At20+4.1, At10+4.1.

4.3 Culture and analysis of small bowel mucosal biopsies in modified DIVAA system with statins

4.3.1 Modified DIVAA system with small bowel mucosal biopsy in mouse

To determine if statin class drugs would increase angiogenesis in an *in vivo* setting, biopsies from a human patient with confirmed CD were suspended in matrigel, secured in a silicone tube and implanted into a host NUDE mouse. Biopsies were mixed with matrigel and 2IU/µl EPO before the mixture was deposited into a 0.24x1cm silicone tube (Sigma) plugged at one end with matrigel. This system was modified from the Directed *In Vivo* Angiogenesis Assay (DIVAA, Trevigen Inc., Gaithersburg, MD, USA) described in section VI of the DIVAA instruction manual. (http://www.trevigen.com/docs/protocol_3450-048-K.pdf?guid=1437592813)

Four such tubes were made, two containing only the biopsy, matrigel and EPO, and each of the other two containing the above contents in addition to either Atorvastatin (10µM)* or Amino-butyro-lactone hydrobromide (ABL) (50µM)* (Sigma). The tubes were then surgically implanted under the back skin of female NUDE mice that had been anesthetized with isoflurane (Sigma), initially in an anesthetizing chamber and then through a mask vaporizer for the duration of the implantation and skin closing. Each mouse was implanted with one tube and then subsequently injected with either Atorvastatin (20mg/kg)** or ABL (100mg/kg)**; the control mice did not receive an injection. The mice were brought out of anesthesia and transferred to cages for a duration of 8 days with free food and water, and a 12 hour light/dark cycle. Reinjections of the drugs for the Atorvastatin and ABL mice were performed at 5 and 7 days, and at day 8 the mice were sacrificed and the tubes were removed and snap frozen. The matrigel plug encompassing the biopsy was removed from the silicone tube and suspended in OCT before storage at -80°C for future analysis.

*Drug concentrations were calculated based on HUVEC cell experiments in section 4.2

**Drug concentrations were based on literature values: ABL (Tessitore 1985) and Atorvastatin (Zhang et al. 2009).

4.3.2 Analysis of DIVAA samples

The frozen biopsy samples were cut as described in section 4.1.2 and were then subjected to Hematoxylin/Eosin staining as described in section 4.1.3 to determine the condition and placement of the biopsy.

5 RESULTS

5.1 Hematoxylin/Eosin stain

The standard H&E histological stain of the cryosliced samples of a matrigel plug containing fragments of the human intestinal mucosal biopsy, revealed the presence and position of the matrigel under the skin of the mouse (Figure 5.1a) and the presence of the biopsy fragments within the matrigel. A higher concentration of cells around the fragments of the biopsy was also observed (Figure 5.1b)

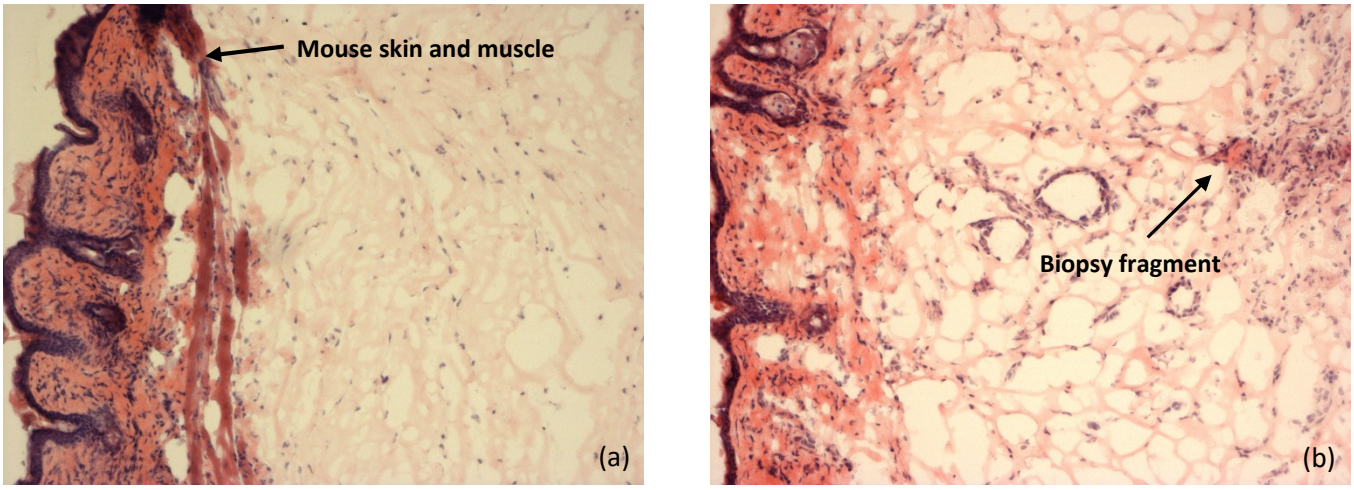
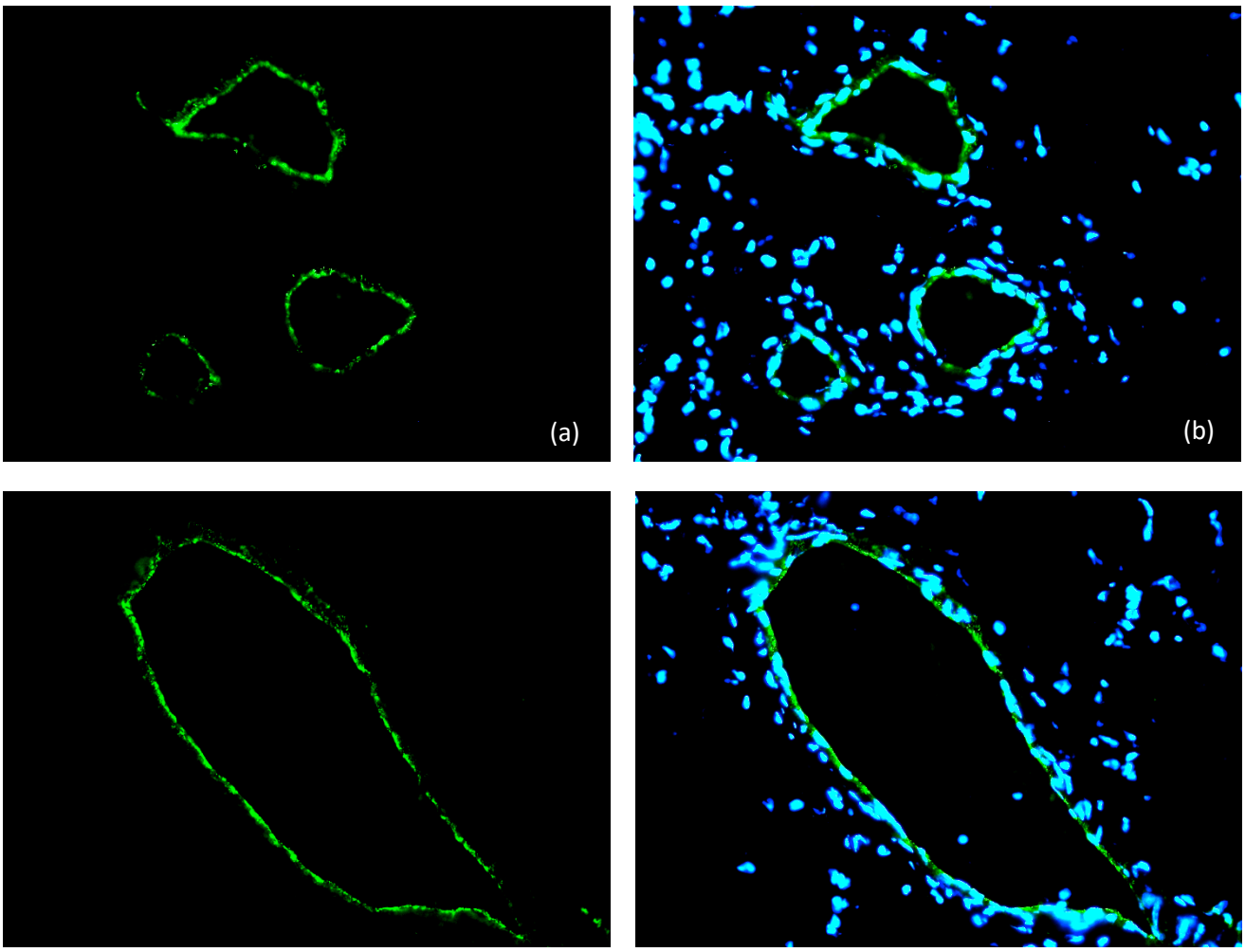


Figure 5.1 The presence of matrigel and biopsy fragments in OCT suspended blocks was detected by HE stain and used to select a set of slides for further analyses. The nuclei of cells are stained purple and any eiosinophilic compounds take on a pink to orange color. Figure (a) shows the skin and muscle of the mouse, and only matrigel with some infiltration of cells. Figure (b) includes the same and additionally, a small fragment of the biopsy with higher cell infiltration.

5.2 vWF stain

The comparison of samples containing fragments of mucosal biopsies from CD patients vs control patients, and stained for the vWF in blood vessels (Figure 5.2a and 5.2b) revealed a significant difference in the size, number and functionality of the vessels between these two groups (Figure 5.2c). This indicates that the fragmented biopsy in the mouse host was able to maintain the feature of reduced vessels formation that is seen in patients during the course of the disease. The functionality of the vessels, as evidenced by the presence of cells inside the vessels, demonstrates that a blood supply to the biopsy is established during the 8 day incubation.



Figures 5.2a and 5.2b Size and number of blood vessels in cryosliced samples containing the biopsies from CD (top 2 panels) and control patients (bottom panels) were determined by an immunofluorescent stain for vWF (green). The cell nuclei (blue) located inside the vessel in the bottom right panel indicates an active vessel complete with blood supply. 10x magnification

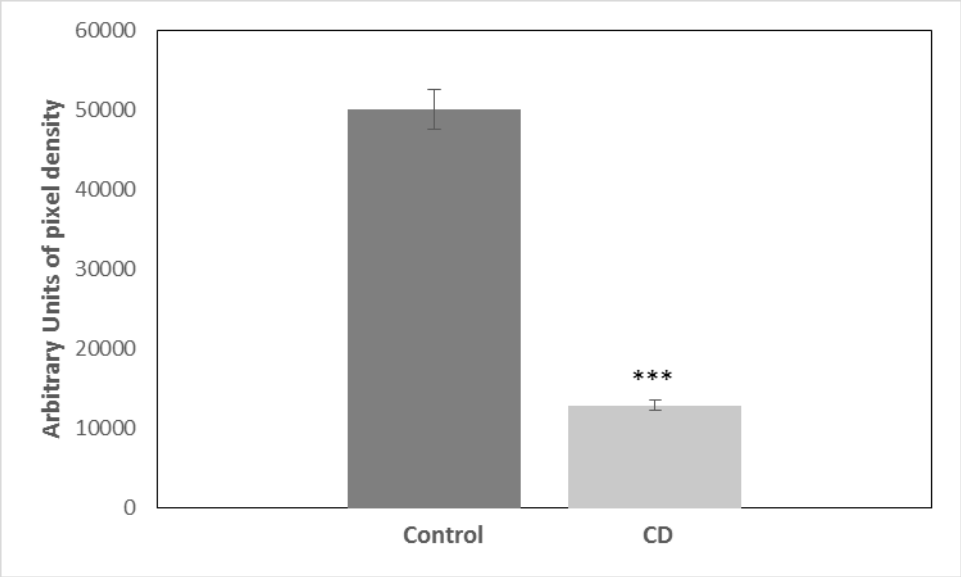


Figure 5.2c Statistical analysis of the number and size of the vessels, quantified in arbitrary units of pixel density, in each sample revealed a significant difference between control biopsies (left) and CD biopsies (right). This gives strong evidence that reduced vessels formation, a feature of CD, is maintained in the host for at least 8 days.

5.3 B and T cell detection

A set of slides from the same series as above, containing both CD and control patients was analyzed for the presence of both B cells and T cells using an immunofluorescent stain for the CD20 marker of B cells and the CD3 marker of T cells. The presence of CD20 (figure 5.3) in several of the CD patient samples but not in the control samples, show that immune cells were not only present at the beginning but also persisted for 8 days. Since these antibodies were specific to the human CD20, the detection of such indicates that the B cells were indeed from the patient biopsy. The antibody used for the detection of the CD3 marker of T cells failed to produce any clear results.

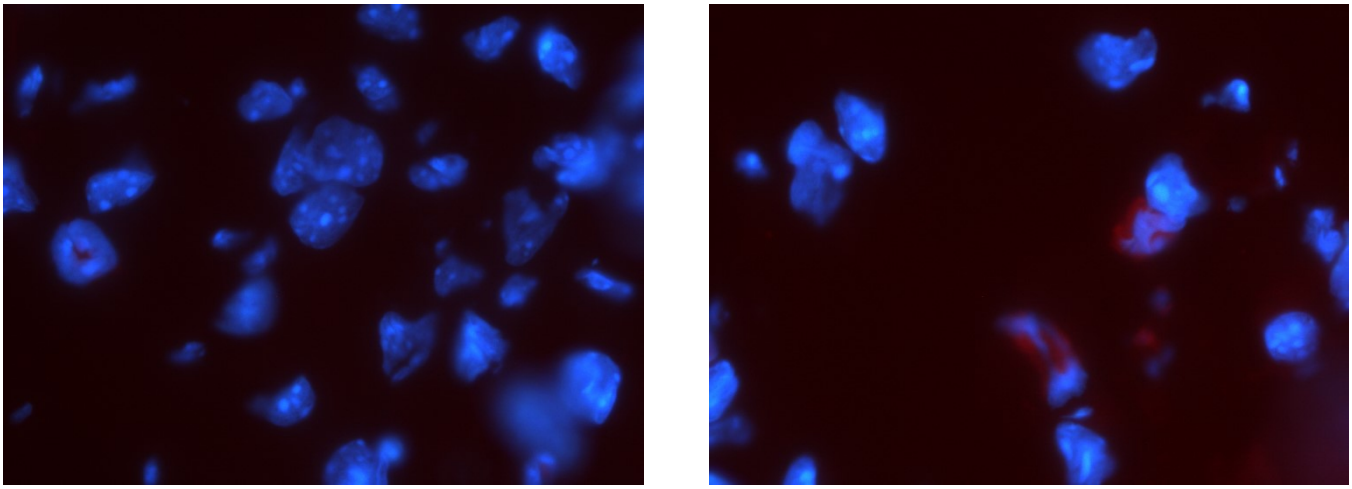


Figure 5.3 Analysis of slides containing biopsies from CD and control patients using immunofluorescent stain of the CD20 marker on B cells (red), overlaid with DAPI stain for cell nuclei (blue). 40x magnification

5.4 IgA/TG2 Colocalization

The colocalization of TG2 and IgA class autoantibodies against it in small bowel mucosa is considered a hallmark feature for CD. A double immunofluorescent stain of IgA and TG2 in a set of samples containing biopsies from CD and control patients (Figure 5.4) revealed that this important feature was indeed preserved during the 8 day incubation in the mice.

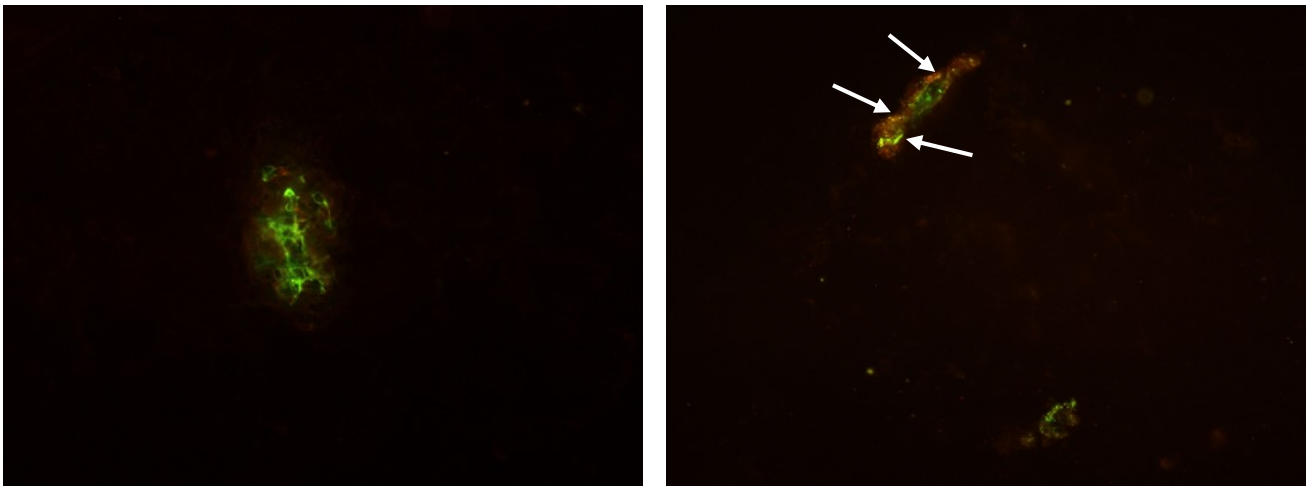


Figure 5.4 CD samples (right) and control samples (left) were analyzed by immunofluorescence to determine the presence of TG2 (red) and IgA autoantibodies (green). Where the two overlap

(yellow/orange) indicates colocalization and is considered an essential feature for the diagnosis of CD, colocalization. 20x magnification

5.5 HUVEC Cell-IQ

5.5.1 HUVEC Cell-IQ with 5 drugs

The percent of wound closure in HUVEC cells subjected to two different concentrations of each of 5 drugs are represented in the table below. The 2 drugs with the highest percent of wound closure were chosen for further experimentation (section 5.5.2 and 5.5.3), namely ABL and Atorvastatin. Their max wound closure percentages were 19.5% and 30.5% respectively.

Average percent closure of HUVEC cells with 5 drugs		
Drug/concentration	**Average % Closure over 72hrs**	**Standard Deviation**
Amino-butyrolactoneHBr/5	NA*	
AminobutyrolactoneHBr/10	19.5	1.77
Pravastatin/5	13	6.72
Pravastatin/10	17.5	6.01
Lovastatin/5	14.5	7.42
Lovastatin/10	13.5	3.18
Mevastatin/5	18.5	4.60
Mevastatin/10	15	3.18
Atorvastatin/5	18.5	3.54
Atorvastatin/10	30.5	6.01
Control	24	5.06

Table 5.5 HUVEC cells were subjected to 5 drugs (names/mM concentrations far left) and tracked using the Cell-IQ system. The average percentage closure of the scratch wound was calculated using Cell-IQ software (middle column). The standard deviation for each is listed on the far right. *Data from ABL/5 pictures was corrupted and not usable

5.5.2 HUVEC Cell-IQ with ABL

The different growth and migration rates of HUVEC cells subjected to two different concentrations of the statin class drug ABL, with and without the 4.1 CD antibody, are shown in figure 5.5. When administered without 4.1 supplementation, and with ABL at concentrations of 50 and 100mM, the scratch wound closed more rapidly, indicating stimulation of the cell migration and growth. ABL at a concentration of 50mM had the most significant increase, showing that the acceleration affect is dose dependent. The 4.1 antibody had a negative effect on the wound closure but not so much as to negate the effect of the drug at 50mM.

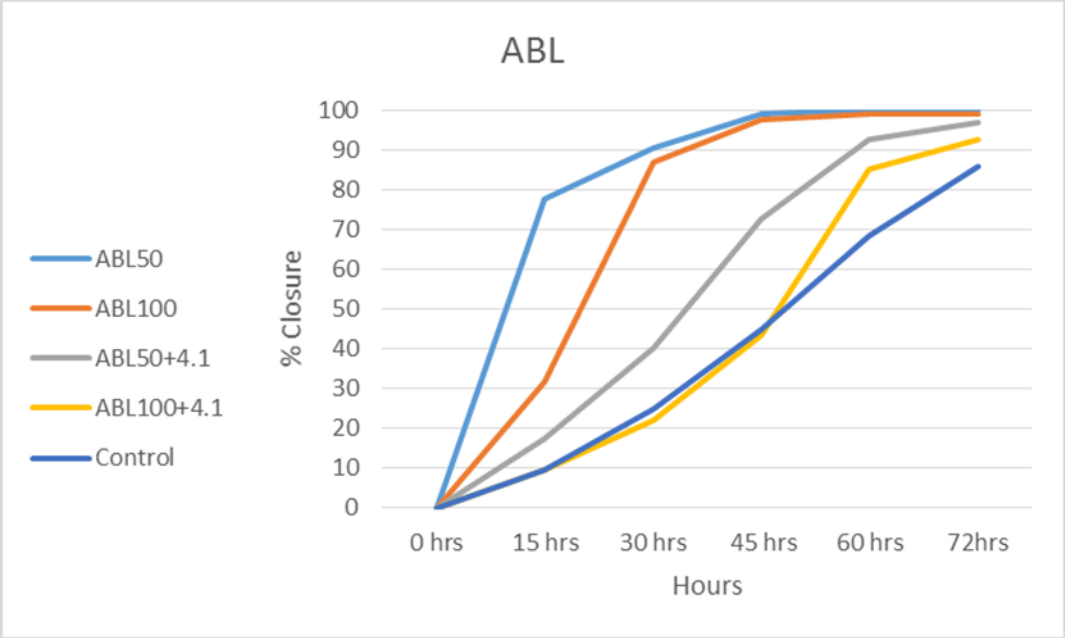


Figure 5.1 The wound closure of HUVEC cells were measured with the Cell-IQ tracking system for 72 hours to distinguish differences between cells grown in the presence of ABL and, with and without the 4.1 antibody, as compared to control cells lacking drug and antibody.

5.5.3 HUVEC Cell-IQ with Atorvastatin

As with the description above, wound closure was measured in the presence and absence of atorvostatin at different concentrations. These measurements did not provide any clear results and in the presence of the antibody, the cell migration appeared more accelerated than in its absence. The At drug at 10mM did not appear to stimulate wound closure beyond control levels, yet in the presence of the 4.1 antibody, accelerated growth compared to the control was seen. The cells in the presence of 20mM At showed accelerated closure for the first 30 hours and then plateaued, while those with the 4.1 continued to migrate more quickly than the control. These results indicate that further testing is needed to obtain more concrete results about the effects of this drug on HUVEC wound closure.

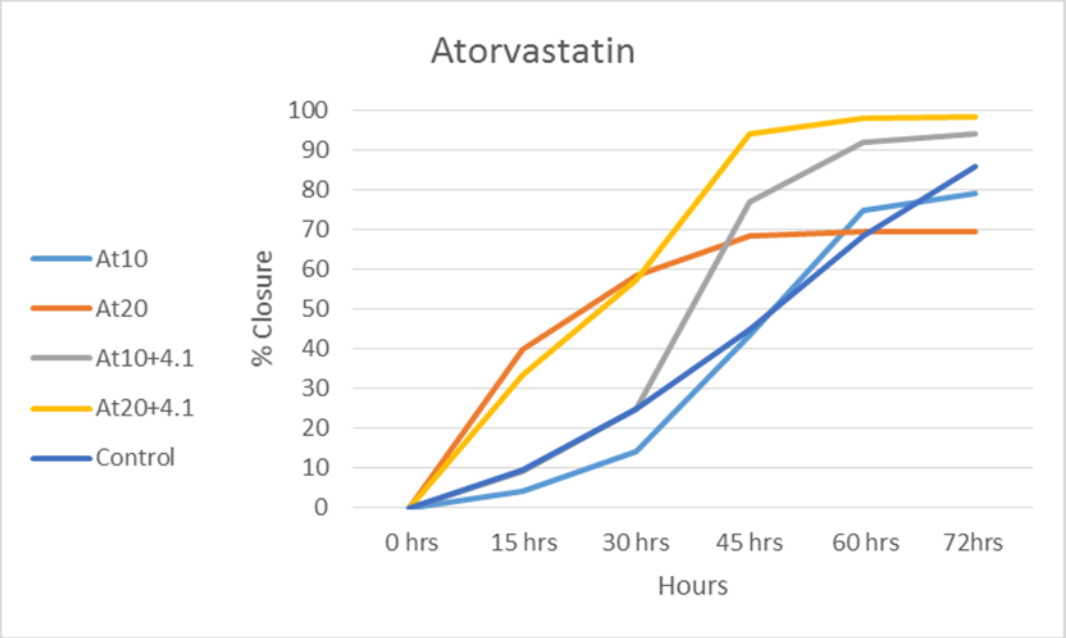


Figure 5.2 Wound closure was tracked with the Cell-IQ system for 72 hours to determine the effect of Atorvastatin at two different concentrations, in the presence and absence of the 4.1 autoantibody. These wound closure rates were compared to a control lacking either drug or antibody, at 15 hour intervals.

5.6 Human biopsy in mouse with statins

A non-CD patient was selected for the experimental control but opted out of the study, leaving only a single CD patient contributing several biopsies. We decided to divide the biopsies evenly between the 4 mice available, 2 non-drug control mice and one mouse with each drug. Following the implantation of the tube containing the biopsy, both of the non-drug control mice ruptured their sutures and displaced their implants. The other two mice that were injected with the drugs maintained their implants for the 8 day incubation period. The biopsies were examined visually when they were extracted from the mice and found to be surrounded by signs of vascularization, with vessels extending into and out of the open end of the tube. However, due to time limitations, full analysis applied to the previous host studies could not be carried out. A repeat of the experiments described above on these intact biopsies, hosted in the mouse with statin drug injections, would contribute to these studies significantly.

6 DISCUSSION

The results of this study clearly indicate that a human biopsy hosted in a mouse can preserve certain features of CD and provide the opportunity to extend the study of human biopsies beyond the usual 48 hours. Essential features of the disease including decreased vessel formation and the hallmark IgA/TG2 colocalization were preserved for the full incubation period. One potential option for this organ culture model is to study pharmaceutical effects on angiogenesis.

It is known that angiogenesis is a key process that is interrupted during the course of CD, important not only for nutrient absorption and other gut endothelial function, but also for the healing that takes place after the removal of gluten from a CD patients diet. For this reason, the presence of the von Willebrand Factor in the biopsy fragments of CD patients compared to the non CD controls, was an important parameter to study. The measurement of this protein provided the first indication that this feature of the disease could be preserved for extended study. In addition to studying the vessel formation in the context of CD, the vascularization of these biopsies also provides an essential blood supply during the incubation period, which is promising for future studies in this system as an organ culture model. This blood supply cannot be provided by a cell culture model and is therefore an advantage of our system over others.

Closely related to the decrease in angiogenesis seen in CD biopsies, is the presence of IgA class autoantibodies linked to TG2, which as discussed in section 2.2, inhibits the sprouting and migration of endothelial and vascular mesenchymal cells, as well as acting pathogenically on the integrity of the endothelium. Although this positive result is encouraging for the overall validity of this model, a greater advantage would come from the study of an intact biopsy instead of a fragmented one. The process of fragmenting the biopsy takes away the opportunity to study the profile of the villi, which are flattened in CD patients and which recover their structure during the healing process. The examination of an intact biopsy was a goal of our study, but one that has yet to be achieved. The results obtained in this study regarding the IgA/TG2 colocalization are preliminary and therefore need further experiments to provide quantitative data about the amount of the antibody and enzyme.

The presence of B cells in the samples were another indication of preservation of the features of CD and since the antibody does not cross react with mouse, it is certain that the few cells that were observed came from the patient biopsies. The lack of plentiful B cells that would normally be seen in diseased tissue could be explained by normal cell migration or by cell death during the 8 day incubation period. This is also a possible explanation for the complete lack of T cells, although the

failure to optimize the immunofluorescent stain also needs to be considered. It would be worthwhile to analyze other tissues in the mouse host to determine if cell migration could explain the absent lymphocytes. Furthermore, the high demand for biopsies may make their analysis at the time of collection difficult, but an analysis of this nature compared to the biopsy after 8 days would prove to be interesting and useful. Given that it is common to take biopsies from each patient under suspect of CD and check for flattened villi to confirm the disease, it would be beneficial to exploit these "zero-hour" samples as well, in association with the medical laboratory. The usual procedure in this case is to take several cryosliced samples from each biopsy, but the remainder of the biopsy is simply stored or used for other tests, and therefore provides an opportunity for further utilization.

After establishing that this model is capable of preserving CD features for the full incubation period, we wanted to investigate whether the decrease in vascularization seen in CD patients could be ameliorated by statin drugs, which had been shown to increase angiogenesis (Gazzerro et al. 2012) and (Skaletz-Rorowski and Walsh 2003). These tests were initially carried out with 5 statins at varied concentrations in HUVEC cells, and then limited to the two drugs with the most promising results, ABL and Atorvastatin. In the 2 statin tests, ABL had a clear accelerating effect on the HUVEC cell migration and growth, which was inhibited by the CD 4.1 epitope but not completely overcome. In our opinion, this data makes ABL a worthy candidate for further study in regards to CD and its effect on angiogenesis during the course of the disease.

The results of the Atorvastatin test were less clear and warrant repeating. The higher concentration of the drug appeared to have an accelerating effect for the first 30 hours, then the cells entered a plateau phase which is consistent with cell death. In this test, the 4.1 epitope had no clear inhibitory effect on the growth and in fact appear to have a positive effect, which is inconsistent with our hypothesis and with the ABL test. The lower concentration of Atorvastatin did not significantly accelerate or decelerate the cell growth or migration, which again did not meet our expectations and was in fact against our hypothesis and the literature. Many studies have shown that At increases cell migration, and although studies with At and the CD 4.1 epitope have not previously been carried out, we expected to see inhibition of migration and growth under these conditions. One possible explanation is a malfunction of the cell tracking system that was used; various pictures taken with this system over the course of the 72 hour experiment were blurry, especially within the Atorvastatin samples and could have cumulatively led to skewed data. Regardless of this possibility, a 4.1 only control would have retrospectively been useful to determine its effect on cells without the presence of drugs, the only control used in these experiments was HUVECs alone.

Despite these unclear results, we decided to proceed with the experiments involving an intact biopsy hosted in a mouse and treated with these same drugs. This decision was based on the success of the ABL results and also on the knowledge that *in vitro* and *in vivo* experiments often yield different results. As this portion of the project proceeded, several issues arose that inhibited us from carrying out the experiments as intended. The first of which was the non-CD control patient we intended to use as a negative control, opted out of the study. This left us with only a single CD patient contributing 2 biopsies, which were divided evenly between the 4 mice. The issue of the control mice displacing their implants could be due to the fact that they were not injected with any drug. We realized later that the DMSO, used to suspend the drugs, can sometimes act as an analgesic, providing a plausible explanation for the fact that the experimental mice did not lose the implants whereas the control ones did (Becker et al. 1969). Following the 8 day incubation with regularly timed drug injections, the implants were harvested from the experimental mice, along with the liver and the spleen. The intention of the study was to analyze the biopsy for the same features as in the previous mouse study including vessel formation, presence of lymphocytes and IgA/TG2 colocalization. We also intended to analyze the liver and spleen for the presence of B and T cells, which could indicate the migration of these cells out of the biopsy, and would account for the low number of these cells in the previous samples. The observed signs of vascularization in and around the implants indicates that the mouse again provided a blood supply to the biopsy. The biopsy, liver and spleen samples were all frozen and stored for analysis but the project terminated at this point due to time constraints.

Despite the many challenges that arose during the course of these experiments, we feel that the results discussed here prove that this model is a valid option for studying CD biopsies and for extending their survival beyond the usual 24-48hr period.

7 CONCLUSIONS

This work explored the possibility of extending the survival time of human small bowel mucosal biopsies to 8 days using a mouse as a host. We concluded that it is indeed possible to extend their survival period outside of the human body and that certain key features of CD were preserved for the entire 8 day incubation time. The measurement of CD vessel formation in and around the human biopsies implanted in mice showed a significant decrease in the size and functionality of the CD vessels compared to the controls. The presence of human B cells and IgA/TG2 colocalization in the CD biopsies after 8 days further demonstrated the capability of this organ culture model to preserve essential features of CD. The secondary objective of this work to study the effects of several statin like compounds on angiogenesis in endothelial cell culture and the CD mucosal biopsies, produced variable yet interesting results. In HUVEC cells, the drug ABL showed an ability to accelerate the cell growth and migration beyond the control cells, even in the inhibiting presence of the CD epitope 4.1. The cells exposed to atorvastatin produced less clear results, still with accelerated growth in the presence of the CD 4.1 epitope, but with more variable growth curves compared to the ABL exposed cells. The application of the drugs to mice implanted with human mucosal biopsies was not fully analyzed and aside from the visual examination of the implants after extraction which showed vessels extending into an out of the biopsy, no data was collected. We conclude that both drugs exhibit interesting potential for angiogenesis studies in the context of CD, but require further investigation.

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